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Short communication

Aliphatic glucosinolate synthesis and gene expression changes in gamma-irradiated cabbage

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ABSTRACT

Glucosinolates, found principally in the plant order Brassicales, are modulated by different post-harvest processing operations. Among these, ionizing radiation, a non-thermal process, has gained considerable interest for ensuring food security and safety. In gamma-irradiated cabbage, enhanced sinigrin, a major glucosinolate, has been reported. However, the molecular basis of such a radiation induced effect is not known. Herein, the effect of radiation processing on the expression of glucosinolate biosynthetic genes was investigated. RT-PCR based expression analysis of seven glucosinolate biosynthetic pathway genes (*MYB28*, *CYP79F1*, *CYP83A1*, *SUR1*, *UGT74B1*, *SOT18* and *TGG1*) showed that *CYP83A1*, *MYB28*, *UGT74B1*, *CYP79F1* and *SUR1* were up-regulated in irradiated cabbage. The content of jasmonates, signalling molecules involved in glucosinolate induction was, however, unaffected in irradiated cabbage suggesting their non-involvement in glucosinolate induction during radiation processing. This is the first report on the effect of gamma irradiation on the expression of glucosinolate biosynthetic genes in vegetables.

1. Introduction

Cabbage (*Brassica oleracea* var *capitata*), a leafy green vegetable of the Brassicaceae family is grown worldwide. Fresh leaves of cabbage are used for preparation in a wide variety of recipes, including delicacies like sauerkraut and kimchi. Vegetables of the Brassicaceae family, including cabbage, have also been extensively investigated recently for their contribution to anticarcinogenic compounds in the diet (Traka & Mithen, 2009). Glucosinolates (GSLs), a group of sulfur containing compounds, are documented to be responsible for the observed chemoprotective activity of these vegetables (Traka & Mithen, 2009). Hydrolysis of the glucose moiety from GSLs by the enzyme myrosinase results in an unstable aglycone that gets converted to a thiocyanate, isothiocyanate or nitrile (Traka & Mithen, 2009). Due to their anticarcinogenic properties, GSLs and their hydrolyzed products have also generated considerable interest as nutraceuticals (Prakash & Gupta, 2012).

There is a limited understanding of the effects of post-harvest storage and processing on the GSL content of Brassica vegetables. Various post harvest processing operations, like refrigeration, shredding and cooking, have been reported to significantly decrease GSL content while UV treatment and storage at ambient temperature has been shown to increase its content in Brassica vegetables (Banerjee, Variyar, Chatterjee, & Sharma, 2014). In a previous study, we reported that radiation processing could result in a significant increase in sinigrin, the major GSL of cabbage, and its hydrolytic product allylisothiocyanate (AITC) (Banerjee et al., 2014). GSLs have been documented to be activated in response to a variety of biotic and abiotic stresses (Variyar, Banerjee, Joseph, & Penna, 2014). A cascade of genes is known to regulate GSL biosynthesis in a stepwise manner. The pathway is regulated by signalling molecules like jasmonates. In *Arabidopsis thaliana*, the elevated expression of genes in aliphatic glucosinolate biosynthetic pathway, as well as transcription factors, such as *MYB28*, *MYB29*, *MYB34*, and *MYB122* involved in their regulation, were found to be induced by jasmonic acid (Guo et al., 2013).

Use of γ-irradiation for eliminating pathogenic and spoilage microorganisms to ensure safety and extension of the shelf life of fresh fruits and vegetables has been extensively reported (Arvanitoyannis, Stratakos, & Tsarouhas, 2009). The FDA (http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm093651.htm) USA, allows the use of γ-radiation up to 4 kGy for microbial decontamination of iceberg lettuce and spinach. Few reports have dealt with the impact of radiation processing on the post harvest physiology of leafy green vegetables at a biochemical, as well as molecular, level. Moreover, γ-radiation can also bring about modulations in metabolic pathways that can influence vegetable processing

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related parameters. We have earlier demonstrated the feasibility of using γ-radiation as an effective post harvest processing method for enhancing the content of sinigrin, the major glucosinolate in cabbage and its hydrolytic product AITC, suggesting that radiation processing can improve the nutritional quality of the vegetable. However, the molecular basis of this radiation induced enhancement has not been studied which may aid in designing strategies for obtaining vegetables with improved functional quality (Variyar et al., 2014). In the present study, the effect of γ-irradiation on transcriptomic changes in glucosinolate biosynthetic genes and content of jasmonates, signalling molecules involved in glucosinolate induction was investigated.

2. Materials and methods

2.1. Plant material

Cabbage (*Brassica oleracea*, BC-79 variety) samples were obtained from field grown plants at Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola with standard agronomic practices of irrigation, fertigation and intercultural operations which were followed to ensure healthy plant growth. Samples were harvested at 30 days after planting when the vegetable weight was approximately 0.5 kg.

Chemicals were purchased from various suppliers: sinigrin, jasmonic acid, methyl jasmonate from Sigma–Aldrich (USA). All solvents were procured from Merck (India) and redistilled before use.

2.2. Irradiation of cabbage samples

Cabbage samples were subjected to γ-irradiation using a ^{60}Co γ-irradiator (GC-5000, BRIT, India, dose rate 4.1 kGy/h) in air to an average absorbed dose of 0.5, 1, and 2 kGy. Dosimetry in the present study was carried out using Fricke dosimeter according to the standard procedure described in ASTM (Designation E2303-11 2011). Dosimeters were placed in the product box (33 cm × 33 cm × 22 cm) at three different layers (front, middle and rear) and at nine different positions of each layer for different time period to map the dose in the gamma chamber. In total, 27 dosimeters were used for a single mapping. The absorbed dose at each point was estimated by the mean value of dose received by all the sets. The minimum dose was received at the centre layer while the maximum dose received at the outer layer. By calculating the D_{max} and D_{min} for all the sets, the DUR (Dose Uniformity Ratio) obtained was 1.12. Uncertainty of the doses was found to be within the limit of ±1%.

2.3. Extraction of glucosinolates

Glucosinolates were isolated from freeze dried cabbage leaves (5 g) using 100 ml of boiling water containing glucotropaeolin (100 μl, 20 mM) as an internal standard for 10 min as reported earlier (Banerjee et al., 2014). The crude aqueous extract thus obtained was successively extracted with ethyl acetate (3 × 30 ml) and *n*-butanol (3 × 30 ml). The different extracts were concentrated to dryness to obtain 10% solution in methanol using a rotary vapor (Buchi Rotavapor-114).

2.4. TLC analysis of glucosinolates

The different extracts as obtained above were subjected to silica gel TLC using *n*-propanol:ethyl acetate:water (7:1:2) as the developing solvent system according to Matsuo (1970). The separated spots were visualized by exposing to iodine vapor and the R_f values of the individual spots were noted. GSLs were identified by comparing the R_f values with standard compounds as well as with literature values. As the *n*-butanol extract was found to be mainly composed of GSLs this extract was chosen for HPLC studies.

2.5. HPLC analysis

The *n*-butanol extract was subjected to HPLC analysis (Jasco HPLC system, Tokyo, Japan) according to the procedure reported earlier (Banerjee et al., 2014). GSLs were identified by comparing their R_t values with standard compounds injected separately under the same conditions as the sample, as well as with literature values. GSLs were desulfated using 10 ml crude aqueous extract (10% solution) to which 500 μl of 0.02 M sulfatase enzyme in aq. NaAcO-AcOH (pH 5) was added and incubated overnight. The resultant mixture was subjected to HPLC analysis as above for further confirmation of GSLs. Owing to desulfation, the peaks corresponding to the GSLs were no longer detected thus acting as a confirmatory test.

2.6. Extraction and estimation of jasmonates

Extraction was carried out according to the method proposed by Xia et al. (2010) with some modifications. 2.5 g of cabbage leaves were ground in liquid nitrogen. Following the addition of 3 ml of methanol, the mixture was stirred vigorously overnight at 4 °C. The slurry was centrifuged at 14,000 rpm (15 min, 4 °C) and the pellet was re-extracted again with 200 μl of methanol. The supernatants were pooled and made to a volume of 10 ml with water containing 500 μg dodecanoic acid as internal standard. The resulting solution was passed through a C18 extraction column (Superclean ENVI-18 SPE, 500 mg). The cartridge was washed with 10 ml of 20% methanol and 10 ml of 30% methanol. Finally, the cartridge was eluted with 10 ml of 100% methanol. The eluent was concentrated in a stream of nitrogen to about 250 μl, weighed and 10% solution was made in methanol.

2.7. HPTLC analysis

HPTLC analysis was performed according to the procedure reported by Dhandhukia and Thakkar (2008). The total extract as obtained in Section 2.6 was subjected to silica gel HPTLC using isopropanol: ammonia: water (10:1:1) as the developing solvent system. The separated spots were visualized by exposing to iodine vapor. Methyl jasmonates and jasmonic acid were identified by comparing their R_f values with values given in literature and from the R_f values of standards spotted separately on the same plate. The area of the individual spots was quantified on a TLC-densitometer (CS9301PC, Shimadzu, Japan) from a standard curve of spot area vs. concentration using different concentrations of standard jasmonates, referred above.

2.8. RNA extraction

RNA extraction was done using Triazol reagent (Sigma, T 9424) as per the manufacturer's instructions. The quantity of RNA was measured using a NanoDrop 3300 spectrophotometer (Thermo Scientific, Waltham, MA) and integrity checked by electrophoresis of total RNA on a 1.2% denaturing agarose gel (Sambrook, Fritsch, & Maniatis, 1989). One μg of total RNA was reverse transcribed with Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.